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Award Number: W81XWH-06-1-0207

TITLE: Biological Impact of Senescence Induction in Prostate Cancer Therapy

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REPORT DATE: January 2007

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-01-2007	Annual	15 Dec 2005 – 14 Dec 2006

4. TITLE AND SUBTITLE

Biological Impact of Senescence Induction in Prostate Cancer Therapy

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0207

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Wisconsin
Madison, Wisconsin 53706-1490

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Recently, it has been recognized that a distinct mechanism of terminal proliferation arrest after chemotherapy involves the reactivation of senescence. However, whether this phenotype occurs in vivo is unclear, as is the biological impact of senescence induction. We have previously identified pathways and genes involved in human senescence that may serve as senescence markers, and have demonstrated that senescence occurs in prostate cancer cell lines after chemotherapy. In this proposal, we will: a) determine whether senescent tumor cells alter the proliferation and invasion of surrounding prostate cancer cells in vitro and in vivo, b) assess for and augment senescence in prostate cancer xenograft models and human tumors, and c) identify novel small molecules that induce senescence in prostate cancer cells. Both in vitro and in vivo approaches using human prostate cancer cells will be utilized to identify and determine the mechanisms underlying senescence. With this data, our understanding of cellular senescence will undergo a quantum leap and permit the translation of this entity both as a marker of response and for directing therapy.

15. SUBJECT TERMS
Androgen receptor, prostate cancer, DNA methylation

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	13	**19b. TELEPHONE NUMBER** *(include area code)*

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Introduction:

Senescence is an irreversible process that limits the lifespan of normal cells. It is believed to represent a tumor-suppression mechanism that is lost during neoplastic transformation. The induction of accelerated senescence, like other damage responses such as apoptosis, is a programmed response to a carcinogenic or biological insult involving multiple molecular pathways. It has recently been appreciated that senescence may also be a cytostatic response *reactivated* in tumor cells in response to chemotherapeutic agents. A limiting factor in identifying and therapeutically exploiting this phenotype has been the lack of molecular markers. In the attached manuscript we present evidence for a panel of senescence-specific molecular markers upregulated in both replicative and induced senescence. We also demonstrate that induction of a senescent phenotype in prostate cancer lines using doxorubicin inhibits growth of untreated cancer cells. It is our **hypothesis** that the therapeutic activity induced by chemotherapeutic agents is due, in part, to a senescence-like program of terminal growth arrest. Furthermore, this phenotype inhibits the proliferation of surrounding cells and its presence may predict tumor response to therapy.

Body:

Task 1: **To determine whether senescent tumor cells alter the growth of surrounding prostate cancer cells *in vitro* and *in vivo***.

a. Co-culture and transwell experiments with ratios of senescent and proliferating cells; Generate senescent DU145 and LNCaP using DAC, doxorubicin and Docetaxel; proliferation and cell count; viablity (months 1-9)
b. Boyden chamber assays using ratios of senescent and proliferating DU145 and LNCaP cells (months 3-12)
c. *In vivo* studies using ratios of senescent and GFP-labeled non-senescent DU145 and LNCaP cells (10 animals per tx group; Total 50 for DU145 and 50 for LNCaP); GFP analysis cell count, BrdU proliferation, PI for viability, TUNEL/PARP for apoptosis. Statistical analyses (months 3-12)
d. If an effect on proliferation or invasion is seen then (months 6-24):
    i. Repeat transwell and Boyden experiments with neutralizing antibodies to IGF receptors 1 and 2, (if stimulatory response) after western confirmation.
    ii. Repeat transwell and coculture experiments with neutralizing antibodies to IGFBP3 and 5(if inhibitory response)
    iii. Selective downregulation of putative effectors in senescent cells using siRNA

Current work on Task 1:

Previous aging studies have attributed a proliferative bystander effect to senescent fibroblasts(1-3). However, in cancer cells that have undergone genomic alteration, whether or not similar bystander effects occur during drug-induced senescence has not been assessed. To address this question, we generated stable GFP-tagged variants of the hormone-refractory DU145 (p53-inactive) and androgen-dependent LNCaP (p53 functional) prostate cancer cell lines using retroviral expression vectors. We developed a model in which these $GFP^{(+)}$ cells are co-cultured with proliferating or senescent

unlabelled cancer cells of the same parent line, and specifically counted by flow cytometry. Low dose (25 nM) doxorubicin in growth medium was used to induce senescence in both cell lines, as previously described and characterized(4). *In vitro*, DU145- $GFP^{(+)}$ or LNCaP-$GFP^{(+)}$ cells were plated with equal numbers of proliferating or doxorubicin-induced senescent untagged cells and cultured in minimal serum-free medium for two and four days.

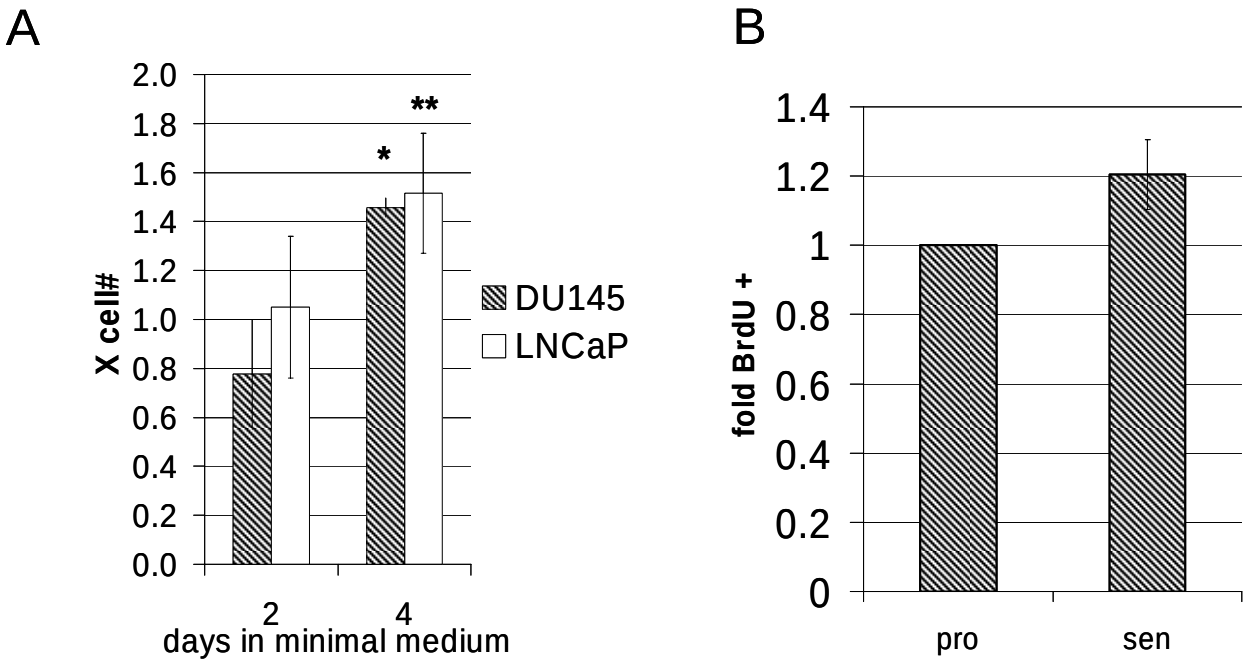

Figure 1: Proliferative bystander effect of drug-induced senescent prostate cancer cells. (A) 50,000 proliferating DU145-$GFP^{(+)}$ cells or 200,000 LNCaP-$GFP^{(+)}$ cells were co-cultured with an equal number of proliferating (pro) or senescent (sen) non-tagged cells in minimal medium for 2 or 4 days. $GFP^{(+)}$-cells were counted by flow cytometry, the total number of cells in each sample calculated, and replicate results were averaged in each experiment. Data were then normalized to the average numbers of cells resulting from proliferating cell co-culture. Results from 4 independent experiments were averaged. These results represent the average fold increase of cell numbers in senescent co-cultures versus proliferative co-cultures. Error bars represent one standard deviation. *: $p<0.0001$; **: $p=0.022$. (B) Average fold increase in BrdU+ nuclei in three independent experiments were averaged and normalized to the averaged results from pro cell co-culture. Error bars represent one standard deviation. $p=0.0033$.

Using both cell lines, cell numbers are similar after two days, but are significantly increased 1.46 and 1.51 fold (respectively) after four days incubation in the presence the senescent cells ($p<0.0001$ and $p=0.022$; Fig 1A). Apoptosis of $GFP^{(+)}$ cells, measured by annexin-V binding and propidium iodide exclusion at each timepoint, is not significantly affected by the presence of senescent cells (<1% in each sample, data not shown), suggesting that the differences observed are not due to effects on cell survival. The increase in DU145 cell number correlated with a 1.2 fold increase (from 16% of the total population to 21%) in BrdU incorporation in the $GFP^{(+)}$ cells exposed to doxorubicin-induced senescent cells ($p=0.0033$; Figure 1B). Holding the overall number of cells constant, we then decreased the fraction of senescent cells 25% and 75% and find that under these conditions there is no significant difference in cell number when compared to cultures containing proliferating cells only (data not shown). In sum, at higher senescent

cell concentrations, drug-induced senescent prostate cancer cells consistently enhance the proliferation of non-senescent cancer cell lines *in vitro,* albeit to a small degree. Senescence bystander effects on proliferation are mediated, in part, by paracrine signaling mechanisms. To determine if secreted diffusible factors are involved in the stimulation of bystander proliferation in our system, co-culture experiments were performed using 0.4 um transwell inserts that prevent cell-cell contact, but expose cells to common medium. Analysis of BrdU incorporation indicates a statistically significant increase in DU145 cell proliferation when co-cultured in transwells with senescent versus proliferating cells ($p<0.0001$; Figure 2A), and similar results are obtained using the LNCaP-based system (20% versus 24% BrdU+, a 1.2 fold increase when cultured with senescent cells).

To identify the protein(s) involved in mediating this response, a panel of secreted growth factor genes previously found to be upregulated in senescent primary prostate epithelial cells(5) were assessed by qPCR in proliferating and senescent DU145 cancer cells. These genes are upregulated in other senescent cell models and have putative

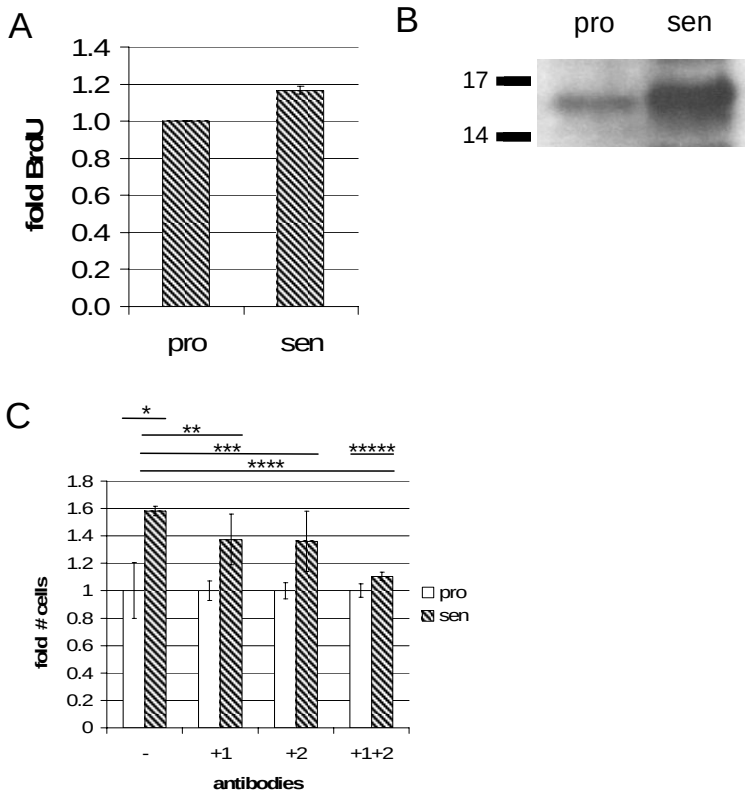

Figure 2: Involvement of secreted proteins in the senescent bystander effect in DU145 cells *in vitro*. (A) BrdU incorporation measured in proliferating DU145-GFP$^{(+)}$ cells co-cultured with proliferating (pro) or senescent (sen) cells in the lower transwell chambers. Average BrdU+ fraction under each condition in three replicates for each experiment were averaged and normalized to the data from cells co-cultured with proliferating cells. The results of 6 individual experiments were then averaged. Error bars represent one standard deviation of 6 individual experiments. $p<0.0001$. (B) IGF2 protein expression in lysates of proliferating and senescent DU145 cells. 20 μg total cell protein in lysates was analyzed by western blotting with anti-IGF2. Specific bands between 10-30kDa (likely cleavage products) were seen to be increased in senescent cells. (C) Blocking of proliferation induced by senescent cells using anti-IGF2 antibodies. Co-culture experiments were performed as in Figure 1, with no addition, addition of anti-IGF2 alone, anti-rabbit secondary antibody alone, or both antibodies together, to minimal medium at total concentrations of 40 ng/ml each (1:5,000 dilutions). These results are representative of three independent experiments. Error bars show one standard deviation in three replicate samples. *: $p=0.008$. **: $p=0.13$. ***: $p=0.16$. ****: $p<0.0001$. *****: $p=0.035$

growth stimulatory effects (1,2,5-7). These numbers were then compared to expression measured in drug-induced senescent cells, and the results of three independent experiments were averaged. Of these, *BRAK*, *Wnt5a* and *IGF2* demonstrate consistent >2 fold increases in transcript expression in senescent DU145 cancer cells (2.5, 3.0 and 3.0 fold, respectively). We chose to focus on *IGF2* since it is reproducibly upregulated in cells induced to senescence(5-7) and is important in prostate cancer proliferation and progression(8). In addition, we find IGF2 protein levels increased in the normalized lysates of drug-induced senescent DU145 cells when compared to proliferating cells (Figure 2B).

To determine whether IGF2 is involved in the proliferative bystander response, we first attempted to use siRNA to block IGF2 expression in senescent DU145 cells. However, no senescent cancer cells survived transfection with the anti-IGF2 siRNA (data not shown). As an alternate approach, we blocked IGF2 activity by co-culturing cells with addition of anti-IGF2 and secondary antibody to co-cultures of senescent and GFP-tagged proliferating DU145 cells. As available IGF2 antibodies do not target the receptor-ligand interface, no significant inhibition of the proliferative response is seen when IGF2 antibody or secondary antibody alone is added to proliferating-senescent co-cultures ($p=0.13$, $p=0.16$ respectively; Figure 2C). Increasing individual antibody concentrations failed to affect proliferation (data not shown). We reasoned that the addition of both anti-IGF2 and a secondary antibody would increase the probability of steric hinderance between ligand and receptor and/or alter the rotational anisoptropy of IGF2 which could interupt ligand/receptor binding. Adding both antibodies to the cells in co-culture results in a significant decrease in the proliferative advantage seen in cultures containing senescent cells compared to cells co-cultured without antibody ($p<0.0001$; Figure 2C). However, while this bystander effect is decreased under these conditions, bystander proliferation remains significantly increased in senescent co-cultures ($p=0.035$), suggesting that additional factors are involved in this effect. Increasing concentrations of the antibody combination decreases constitutive proliferation of both senescent and proliferating co-cultures (data not shown) and may reflect a pivotal role of IGF2 in DU145 growth and survival. These results suggest that increased IGF2 secretion by drug-induced senescent cancer cells is involved, in part, in the enhanced proliferation of co-cultured non-senescent DU145 cells. Previous work in normal senescent fibroblasts had identified the involvement of MMP3(3) and amphiregulin(1) in this proliferative effect. While our previous data does not implicate the involvement of these genes in this bystander effect, our data can neither rule out their involvement in these phenomena we observe.

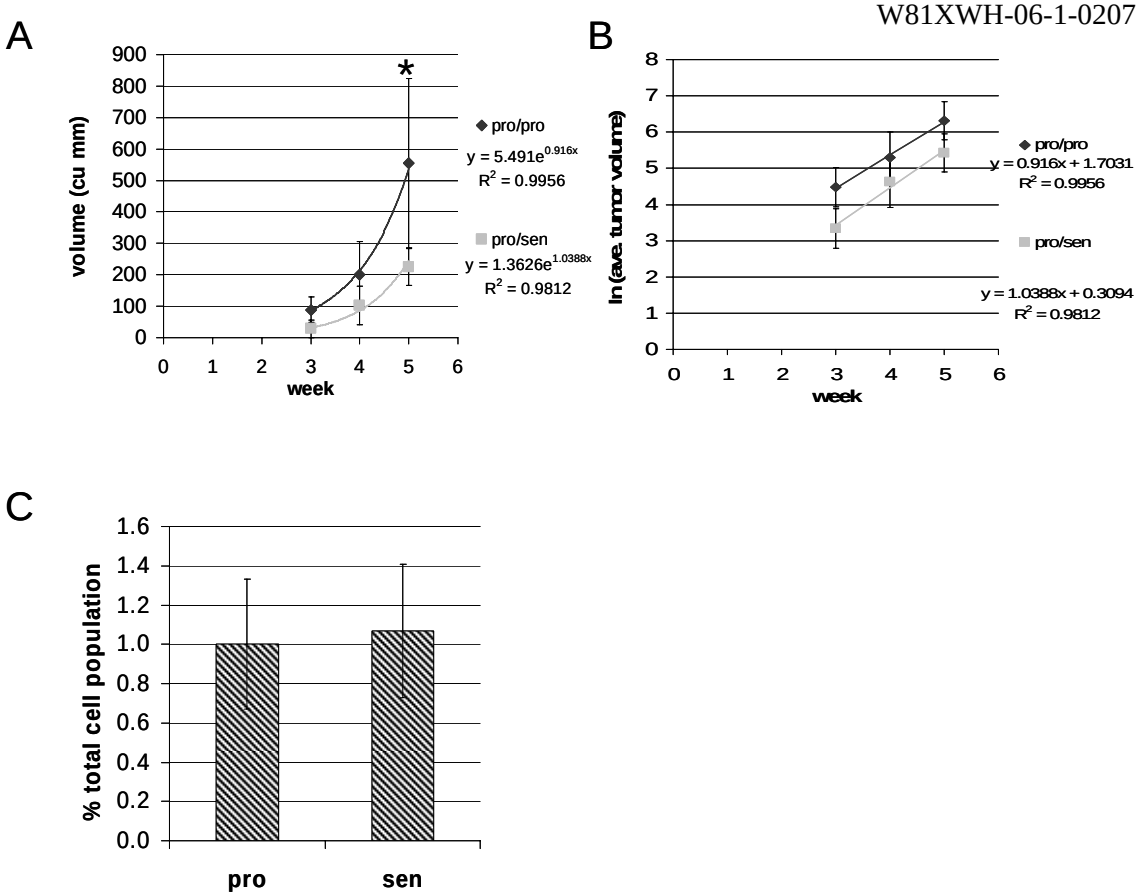

Figure 3: Proliferation of prostate xenograft cells co-injected with proliferating or senescent cells. $0.5x10^6$ cells each of DU145-GFP$^{(+)}$ cells and proliferating (pro) or senescent (sen) DU145 cells were injected into the subinguinal fat pad of male nude mice and allowed to develop into tumors over 5 weeks. These results represent two independent experiments. (A) Tumor volumes were measured weekly once detectable, from 3 to 5 weeks after injection. Error bars represent one standard deviation in 10 replicates. Data was modeled to an exponential fit, and equations are included in the figure legend. $p<0.001$. (B) The natural log (ln) of average tumor size was calculated and plotted versus time. The resulting data were fit by linear regression, and equations are included in the figure. Error bars represent one standard deviation of the natural log of 10 individual tumor sizes. (C) At 5 weeks, after intraperitonal injection of BrdU and 2 hr recovery, tumors were harvested from mice, GFP$^{(+)}$ cells were recovered by florescence activated cell sorting, fixed, and subsequently analyzed for BrdU incorporation and nuclear DNA content. Numbers were normalized to those resulting from proliferating cell co-culture. Error bars represent one standard deviation in ten replicates. $p=0.69$.

Having observed senescence bystander proliferation *in vitro*, we questioned how senescence induction in half of a population of prostate cancer cells would affect bystander cell growth *in vivo*. Extending our model, xenograft tumors were established by co-injecting $0.5x10^6$ DU145-GFP$^{(+)}$ proliferating cells with an equal number of unlabeled proliferating or senescent DU145 cells into the subinguinal fat pad of nude mice. This experiment could not be replicated using LNCaP cells, as xenograft establishment with these cells requires the introduction of supplemental growth factors which confound the experiment. DU145 tumors were detected after 2 weeks, and tumor dimensions measured at 3, 4 and 5 weeks after injection. The average volume of tumors established with or without senescent cells was calculated for each timepoint, and the data fit to exponential equations. Control animals in which only senescent cells are injected do not develop detectable tumors through the course of this experiment (data not shown). Xenografts containing only proliferating cells grew significantly larger than those containing senescent cells in five weeks ($p<0.001$), correlating with the greater

number of proliferating cells initially injected (Figure 3A). However, the average rate of tumor growth (0.916 versus 1.038 in the fit equations of Figures 3A and B) is not significantly affected by the co-injected senescent cells. Plotting the natural log (ln) of the average tumor volume over time additionally illustrates differences in average tumor size but similarity of growth rates in both sets of tumors (Figure 3B). To further validate this observation we labeled tumors with BrdU prior to harvest to measure proliferation in the $GFP^{(+)}$ tumor cells after sorting. DU145-$GFP^{(+)}$ cells from tumors established with or without senescent cells contain similar sized fractions of proliferating cells, approximately 5% of the total cell population, with no significant differences (Figure 3C). These results indicate that there is no significant effect of drug-induced senescent cancer cells that enhance the long-term proliferation of bystander tumor cells *in vivo*.

Specific Aim 2: **To assess for and augment senescence in prostate cancer xenografts and human tumor tissues.**

a. Generate Du145 and LNCaP xenografts in nude mice (months 6-24)
b. Treat with Docetaxel or doxorubicin and harvest at 3 intervals (3 intervals X 10treated/10control per xenograft line = total 60 for DU145 and 60 for LNCaP). GFP analysis cell count, BrdU proliferation, PI for viability, TUNEL/PARP for apoptosis (months 12-30)
c. QPCR and immunohistochemistry for senescence markers (months 12-36)
d. Analysis of human neoadjuvant tissues (10 treated/10 untreated per trial X 2). QPCR and immunohistochemistry for senescence markers (months 24-36)
e. Statistical analyses and correlation with proliferation

Current work on Task 2:
We are currently setting up the mouse experiments for this Aim.

Specific Aim 3: **To screen for small molecules capable of inducing senescence.**

1. Generate senescence reporter construct using CSPG2 and stably transfect prostate cancer cell lines DU145 and immortalized human prostate epithelial cell line HPV16E7. Select and test reporter. (months 1-6)
2. Optimization of detection conditions (months 6-12)
3. Screen 500 compounds with DU145 to gauge appropriate concentration
4. Screen full 16,000 compound library (months 12-18)
5. Secondary analyses of 25 most active compounds in other prostate cancer cells lines including QPCR for senescence markers, morphology, cell cycle arrest and SA B galactosidase staining. (months 18-30)

Current work on Task 3:

We adapted previously published protocols for identifying senescent cells to develop a high throughput whole cell phenotypic assay to identify senescence inducing compounds in a 96 well format. The DU145 cell line was chosen for this screen because

they represent advanced hormone-refractory prostate cancer that additionally express mutant inactive p53, attach well to cell culture surfaces, withstand repeated washes, and develop a strong and distinct senescence phenotype. 10,000 cells are plated in 100 l growth medium per well to allow subsequent proliferation of unaffected cells. After an overnight incubation, cells are treated with compounds and incubated 3 days after which they are fixed and stained overnight for SAB-gal activity and preserving cellular morphology. Cells are washed and incubated with the fluorescent DNA-binding dye Hoechst 33342 in PBS, followed by another wash and storage in PBS. Hoechst 33342 350/460 fluorescence was then measured in each well using a high throughput plate reader and used to differentiate wells in which compounds had no effect and continued proliferation, or wells where cells either arrested or apoptosed. In control experiments using this method to assess the dose response of doxorubicin, previously characterized as a strong inducer of senescence, Hoechst 33342 fluorescence measurements of senescent cells treated with 25nM doxorubicin were significantly less than that of cells that were not treated (Fig 4).

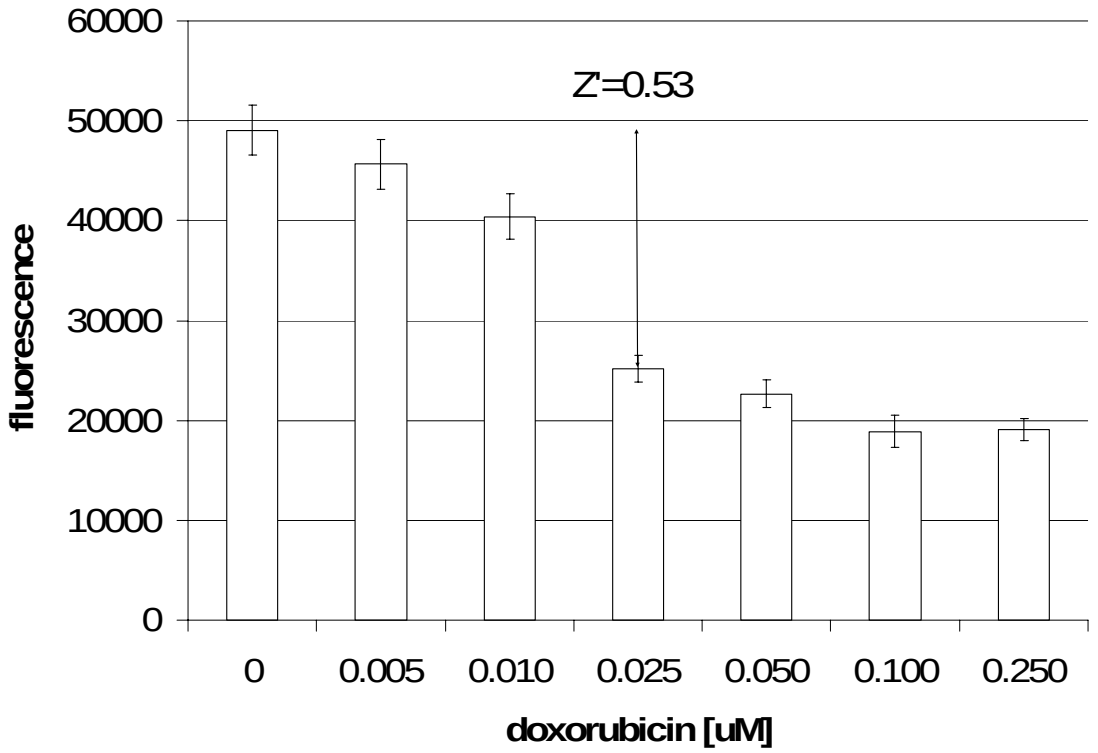

Figure 4: Induction of senescence using increasing doses of Doxorubicin in Du145 cells correlated with PI staining. This generates a distinct separation between proliferating and senescent cells.

These measurements were used to calculate a Z'-factor of 0.53, validating this method as a reliable indicator to differentiate proliferating and senescent cells in a high throughput assay. This proved more reliable than initial attempts to develop this assay based on increased expression of senescence reporter genes due to difficulties differentiating increased expression in few senescent cells and basal expression in numerous cells (data not shown). This however does not differentiate between senescent

and apoptotic cells treated with increased concentrations of doxorubicin, which also have low fluorescence. To differentiate these populations, wells with significantly less fluorescence would be visually inspected and determined whether the cells were apoptotic or arrested but viable. Where cells were not apoptotic, senescence induction is visually assessed based on cellular morphology and increased SAB-gal activity. Selected compounds would then be selected using secondary assays that further validate senescence induction.

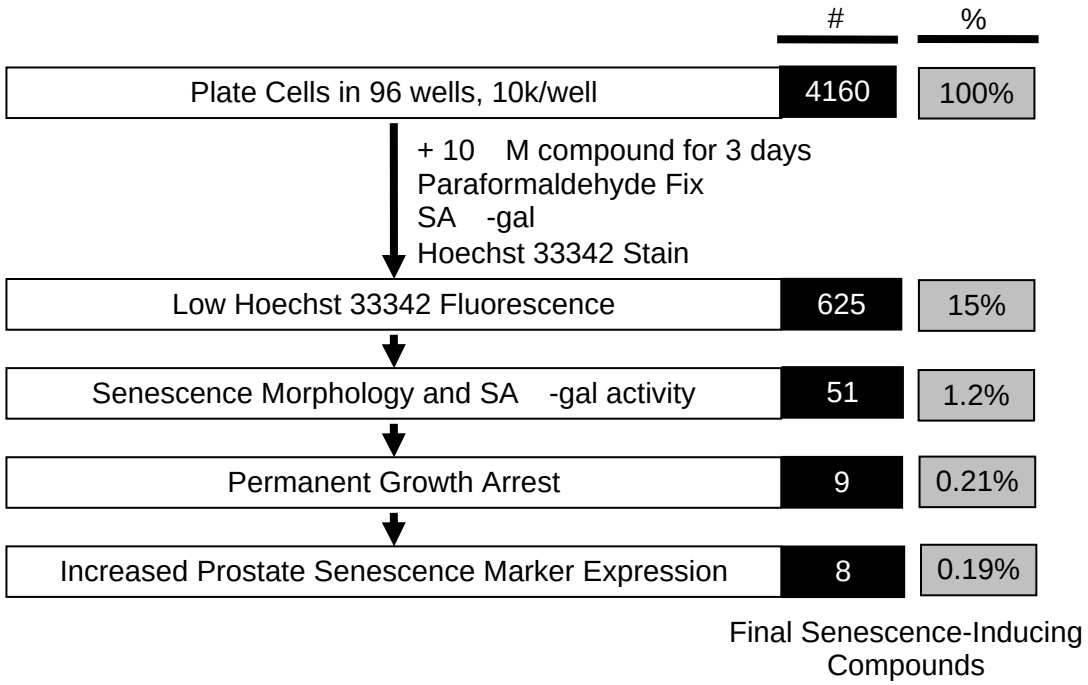

Figure 5: Schematic of results of senescence assay using the current approach.

To test and validate this assay, we chose to screen a small pilot library of known bioactive compounds and natural products (KBA library) that contains structurally diverse characterized compounds, drugs, pollutants and naturally occurring extracts. For the purposes of the screen, wells with fluorescence >1 standard deviation less than the average of all data constituted an initial "hit", allowing a relatively less stringent initial selection of compounds. This resulted in 625 candidate compounds (Fig 1c), approximately 15% of the library, which were visually and arbitrarily scored by two separate individuals to identify potential senescence inducers, further selecting 51 candidate compounds. These were then assessed to determine whether compounds induced a permanent growth arrest or proliferation recovered in cells after removal of the compound by adapting the above assay to test candidate compounds in duplicate wells, reducing the number of candidate compounds to 9 (Figure 5).

With fewer samples, candidate compounds can be assessed using more detailed analyses of senescence induction using qPCR to measure expression of previously identified senescent marker genes. DU145 cells were seeded into duplicate wells of a 96

well plate, incubated overnight, and treated with 10 M of each candidate compound, 25nM doxorubicin as a positive control, or left untreated. After 3 days incubation, RNA was isolated, reverse transcribed to cDNA and gene expression was measured by qPCR using specific primers for 18S rRNA and the previously identified senescence markers cspg2, IGF2, BRAK and GLB1. After first standardizing expression to 18S expression levels, data was normalized to expression in untreated proliferating cells. This shows that 8 of 9 candidate compounds induced increased marker gene expression at levels exceeding that induced by doxorubicin, validating the senescence-inducing activity of these compounds. This experiment was reproduced using the hormone-dependent LNCaP prostate cancer cell line to validate that each compounds' senescence-inducing activity is not specific to DU145 cells. These results again show significantly increased expression of senescence marker genes in cells treated with each of the 8 remaining compounds.

Key Research Accomplishments:

- Senescence induces a bystander effect in vitro but not in vivo
- *In vitro* senescence is mediated by the IGF axis
- Development of a novel, whole-cell senescence screen
- Screening of a 4,100 compound library
- Identification of 8 senescence-inducing compounds

Reportable outcomes:

- Presented abstract of "Drug-Induced Senescence Bystander Proliferation In Prostate Cancer Cells *In Vitro* and *In Vivo"* at AUA meeting in Atlanta, GA (5/2005)
- Presented abstract of "Drug-Induced Senescence Bystander Proliferation In Prostate Cancer Cells *In Vitro* and *In Vivo"* at AACR meeting in San Francisco, CA (12/2006; won travel award for this meeting)
- Presented abstract of "High-Throughput Whole-Cell Screen Identifies Novel Compounds that Induce Senescence in Prostate Cancer Cells" at AACR meeting in San Francisco, CA (12/2006)

Conclusions:

We conclude that while drug-induced senescent cells stimulate the proliferation of surrounding cancer cells *in vitro*, this does not significantly affect the long term growth of bystander cells that might escape senescence induction. These data support further development of senescence-induction strategies for cancer treatment. Furthermore, the above results validate the ability of this high-throughput assay to identify senescence induction. It provides a tool to develop novel senescence-inducing compounds for prostate cancer therapy, as well as providing further insight into mechanisms of senescence induction.

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